Structure-activity study of a laminin α1 chain active peptide segment Ile-Lys-Val-Ala-Val (IKVAV)

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Abstract The IKVAV sequence, one of the most potent active sites of laminin-1, has been shown to promote cell adhesion, neurite outgrowth, and tumor growth. Here we have determined the structural requirements of the IKVAV sequence for cell attachment and neurite outgrowth using various 12-mer synthetic peptide analogs. All-L- and all-D-IKVAV peptides showed cell attachment and neurite outgrowth activities. In contrast, all-L- and all-D-reverse-sequence peptides were not active. Some of the analogs, in which the lysine and isoleucine residues of the IKVAV peptide were substituted with different amino acids, promoted cell attachment, but none of the analog peptides showed neurite outgrowth activity comparable to that of the IKVAV peptide. These results suggest that the lysine and isoleucine residues are critical for the biological functions of the IKVAV peptide.

Key words: Laminin; IKVAV; Synthetic peptide; D-Amino acid; Cell attachment; Neurite outgrowth

1. Introduction

Laminins comprise a family of large heterotrimeric basement membrane glycoproteins, which have diverse biological activities including promoting cell adhesion, growth, migration, neurite outgrowth and differentiation, and influencing the metastatic potential of tumor cells [1–3]. Several laminin isoforms have been identified with at least eight genetically distinct chains [4]. Laminin-1 consists of three chains designated α1, β1 and γ1 chains, and has a cruciform shape with one long and three short arms when examined by electron microscopy [3,5]. Several active sequences of laminin-1 have been identified using synthetic peptide approaches [6–13]. An Ile-Lys-Val-Ala-Val (IKVAV) sequence located on the C-terminal end of the long arm of the laminin α1 chain was found to be active in promoting cell adhesion, neurite outgrowth, angiogenesis, collagenase IV production, and tumor growth [10,14–17]. Cell surface IKVAV sequence binding proteins have been studied and a 110 kDa membrane-associated laminin-binding protein from brain was previously shown to bind to the IKVAV site [18]. Recently β-amyloid precursor protein was found to specifically bind to the IKVAV peptide [19]. Additional IKVAV-specific binding proteins likely exist.

It is important to identify the structural requirements of the active IKVAV peptide for a better understanding its biological functions and specific interactions with its receptors or binding proteins. Previously, we showed the all-D-configuration segment containing the IKVAV sequence of laminin α1 chain had similar cell attachment and tumor growth activities to the all-L-peptide in vitro and in vivo [20]. These data suggested that the conformational status of the IKVAV domain is a contributing factor in determining the biological activity but that there is no strict requirement for a specific chirality. Furthermore, such data suggested that there is a likely sequence specificity to the IKVAV region.

In the present paper, we describe the structural requirements of IKVAV containing peptides of the laminin α1 chain for cell adhesion and neurite outgrowth using various synthetic peptide analogs. Peptide sequences from related laminin a chains containing the corresponding region were also evaluated. The data show cell attachment and neurite outgrowth activities have different structural requirements suggesting multiple cellular receptors.

2. Materials and methods

2.1. Synthesis of peptides and laminin-1

All peptides were synthesized manually by 9-fluorenylmethyloxycarbonyl (Fmoc) based solid-phase methods. The respective amino acids were condensed manually in a stepwise manner using the Tris-(alkoxy)-benzylamine resin [21]. For condensation, diisopropylcarbodiimide-*N*-hydroxybenzotriazole was employed, and for deprotection of Fmoc groups, 20% piperidine in dimethylformamide was employed. The following side chain protecting groups were used: Asn, trityl; Asp, Glu, and Ser, t-butyl; Arg, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Lys, t-bulyloxycarbonyl. The protected peptide resins were deprotected with a two-step procedure, which consisted of consecutive treatments with trifluoroacetic acid (TFA)-thioanisole-*m*-cresol-ethanedithiol-H_2O (80:5:5:5:5, by volume) at 20°C for 2 h followed by 1 M trimethylsilyl bromide-thioanisole in TFA in the presence of *m*-cresol and ethanedithiol at 4°C for 1 h [22]. Resulting crude peptides were purified by reverse-phase high performance liquid chromatography (HPLC) (using a Vydac 5C18 column with a gradient of H_2O/acetonitrile containing 0.1% TFA). Purity and identity of the synthetic peptides were confirmed by analytical reverse-phase HPLC and by amino acid analysis. Amino acid analyses were performed at the Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan.

Mouse laminin-1 was prepared from the Engelbreth-Holm-Swarm tumor as described previously [23].

2.2. Cells and culture

PC12 cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) containing 7.5% fetal bovine serum, 7.5% horse serum, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. These cells were generously provided by Gordon Guroff (NICHD, NIH). For the cell attachment and neurite outgrowth assays, the PC12 cells were washed free of serum in 50% DMEM and 50% Ham's F12

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(DMEM/F12) containing 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were then cultured in the serum-free DMEM/F12 for the duration of the attachment and neurite outgrowth assays.

2.3. Cell attachment assay using synthetic peptides and laminin-1

For attachment assays, the peptides were dissolved at a concentration of 0.5 mg/ml in a 10% acetic acid solution. The indicated amounts of peptides were then added to the 16 mm diameter wells of a 24-well plate (Corning, Corning, NY). The peptides were then allowed to dry overnight. Next, a solution of 3% bovine serum albumin (BSA) was added to each well. After one hour, the BSA was removed and 50,000 cells were added to each well in serum-free DMEM/F12. The cells were then incubated at 37°C in a CO_2 incubator. After one hour, the medium and unattached cells were removed by aspiration and the attached cells were fixed and stained with Diff Qick (Baxter Scientific Products, Miami, FL). The percent of attached cells was determined by counting the center 2 mm^2 field in each of triplicate wells. The 2 mm^2 field represents 1.6% or 1/64 of the well. Therefore the average number of cells in the 2 mm^2 field was divided by the total number of cells added to each well and this number was multiplied by 100 to determine the percent of cells attached.

2.4. Neurite outgrowth assay

For the neurite outgrowth assays, 16 mm diameter wells of a 24-well plate were coated with the indicated amounts of peptides as described above. After the peptides dried, the wells were rinsed with DMEM/F12 to remove residual acid. PC12 cells were primed with 100 ng/ml of nerve growth factor 24 h prior to their use. After priming, the PC12 cells were washed free of serum and seeded into the wells in serum-free DMEM/F12. The cells were then incubated at 37°C in a CO_2 incubator for 24 h and the cells were fixed and stained as described above. In each of triplicate wells, a total of 100 cells was viewed and the percent which had neurites that extended twice the cell diameter or greater was determined and averaged for each peptide amount tested.

2.5. Coating efficiency

Coating efficiency of the synthetic peptides were determined as shown previously [20]. Laminin-1 (5 μg/well) and the synthetic peptides (500 mg/well) were each added to 6 wells of the 24-well dishes and dried at room temperature overnight. Half of peptide-coated wells were gently washed three times with PBS (1 ml each). Coated peptides were twice extracted with PBS (1 ml each) containing 3% Triton X-100 and with PBS (1 ml). Fluorescamine in acetone (50 μl, 1 mg/ml) was added to the extracted solutions (total 3 ml), and fluorescence emission at 475 nm was determined using a Perkin-Elmer Model LS-5B Luminescence Spectrometer with excitation at 390 nm. Coating efficiencies (%) were determined using fluorescence intensity of PBS-washed samples relative to non-washed samples from the wells.

3. Results and discussion

Analog peptides were designed based on an IKVAV sequence containing 12-mer peptide (LAM-L: mouse laminin α1 chain residues 2097–2108 [24]) (Table 1). All-D-peptides containing IKVAV sequence (LAM-D) and all-L- and all-D-reverse-sequence peptides (LAM-LR and LAM-DR, respectively) were prepared. As a control, a peptide containing a randomized IKVAV region was also prepared. Lysine position substituted analogs, in which the lysine residue in the LAM-L peptide was replaced with either arginine which is more basic or glutamic acid which has a negative charge (LAM-KR and LAM-LE, respectively), were prepared. Isoleucine position substituted analogs, in which the isoleucine residue in the LAM-L was substituted with either leucine, norleucine, valine and alanine (LAM-IL, -IX, -IV and -IA, respectively), were synthesized (Table 1). Homologs and different species laminin peptide segments containing the corresponding region of LAM-L were prepared using mouse laminin α2 chain, human laminin a3 chain and *Drosophila* laminin a chain sequences [25–27] (MER-12, KAL-12 and DOR-12, respectively).

Cell attachment and neurite outgrowth activities of reverse-sequence and mirror image compounds of the IKVAV peptide (LAM-L) were examined using rat pheochromocytoma PC12 cells (Fig. 1A,B). Previously, the reverse-sequence of the Arg-Gly-Asp (RGD) peptide from fibronectin was shown to inhibit cell adhesion on the cell binding domain of fibronectin as well as the native RGD peptide [28]. The reverse-sequence laminin-derived peptide containing all-L- and all-D-amino acids (LAM-LR and LAM-DR, respectively) did not showed cell attachment activities. The all-L- and all-D-peptides (LAM-L and LAM-D, respectively), which were previously shown to promote cell adhesion and tumor growth [20], showed cell attachment activity for PC12 cells as expected (Fig. 1A). LAM-D had reduced neurite outgrowth promotion activity with approximately 60% of the activity of that observed with the potent peptide (Fig. 1B). These results indicate that both all-L- and all-D-containing IKVAV peptides can bind to cell surface receptors or binding proteins, but that the all-L- and all-D-VAVKI-containing peptides (a reverse-sequence of IKVAV peptide) cannot interact. These results suggest that the structural requirements of IKVAV peptide for interaction with its cell surface receptor or binding proteins are specified by the specific primary structure. Cell attachment and neurite outgrowth promotion activities of laminin-1 were considerably higher than

Table 1
Synthetic peptides and their biological activities with PC12 cells

Peptide	Sequence	Cell attachment	Neurite outgrowth
LAM-L	AASIKVAVSADR	++	++
LAM-D	AASIKVAVSADR	++	+
LAM-LR	RDASVAVKISAA	−	N.D.
LAM-DR	RDASVAVKISAA	−	N.D.
LAM-RM	AASVVIAKSADR	−	N.D.
LAM-KR	AASI**R**VAVSADR	++	+
LAM-KE	AASI**E**VAVSADR	−	N.D.
LAM-IL	AAS**L**KVAVSADR	+	−
LAM-IX	AAS**X**KVAVSADR	++	+
LAM-IV	AAS**V**KVAVSADR	++	−
LAM-IA	AAS**A**KVAVSADR	−	N.D.
MER-12	ANSIKVSVGGGG	++	++
KAL-12	AASKVAVPMRFN	−	N.D.
DOR-12	ANSIKVGVNFKP	++	++

LAM-L, all-L-configuration mouse laminin α1 chain peptide segment (residue 2,097–2,108 [24]); LAM-D, all-D-configuration LAM-L; LAM-LR, reverse-sequence LAM-L; LAM-DR, reverse-sequence LAM-D; LAM-RM, all-L-configuration laminin segment with the IKVAV segment scrambled; LAM-KR and -KE; the lysine in LAM-L is replaced with arginine or glutamic acid, respectively; LAM-IL, -IX, -IV and -IA; the isoleucine in LAM-L is replaced with leucine, norleucine, valine and alanine, respectively. MER-12, mouse laminin α2 chain peptide segment (residue 2,137–2,148 [25]); KAL-12, human laminin α3 chain peptide segment (residue 763–774 [26]); DOR-12, *Drosophila* laminin a chain peptide segment (residue 2,568–2,579 [27]). D-configuration amino acids are underlined. Replaced amino acids are written in bold type. All peptides have C-terminal amides. Cell attachment and neurite outgrowth activities (Figs. 1 and 2) are summarized: ++activity comparable to that of LAM-L; +active but weaker than LAM-L; −, no activity; N.D., not determined.

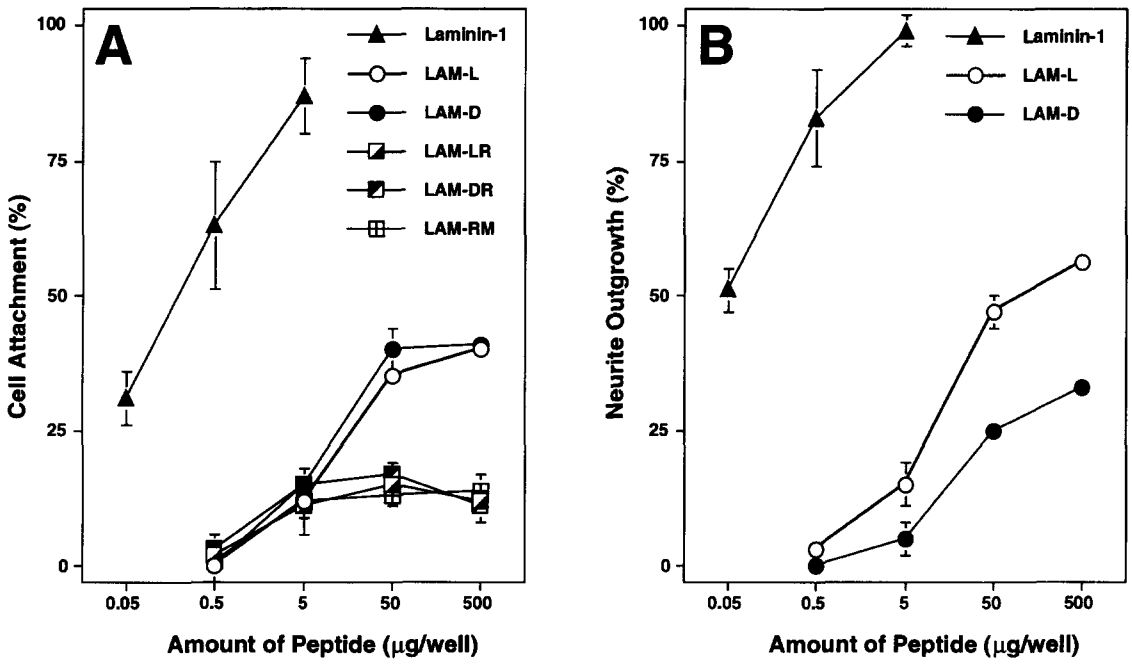

Fig. 1. Attachment (A) and neurite outgrowth promotion (B) of PC12 cells on various synthetic peptides and on laminin-1. Each value represents the mean of three separate determinations ± S.E. Duplicate experiments gave similar results. Coating efficiencies of laminin-1 (5 μg/well) and the synthetic peptides (500 μg/well) were 43.8% (laminin-1), 1.9% (LAM-L), 1.4% (LAM-D), 3.4% (LAM-LR), 2.8% (LAM-DR) and 0.9% (LAM-RM).

those of LAM-L on a molar basis. This is not surprising because small peptides usually have less rigid conformations and laminin-1 can also interact with cell surface receptors through ancillary sites.

Next we tested the importance of the lysine position in the IKVAV sequence for biological activities using single amino acid substituted analogs, in which the lysine was replaced with Arg or Glu (LAM-KR and LAM-KE, respectively). The arginine residue has the strongest basic charge among the amino acids and is present in the active sequences of many extracellular matrix proteins [7,29]. In addition, arginine residues likely play an important role in interaction with integrins [29]. The arginine-containing IKVAV substituted peptide, LAM-KR, showed cell attachment comparable to that of LAM-L, but the glutamic acid substituted peptide, LAM-KE, did not show activity (Fig. 2A). However, LAM-KR could not promote neurite outgrowth as well as that of the IKVAV peptide (LAM-L) showing about 60% of the activity (Fig. 2B). These results suggest that the contribution of the lysine residue of the IKVAV sequence is critical for biological activity. A positive charge in this location is important for cell attachment and is replaceable with other basic amino acids such as an arginine. The lysine residue is specifically required for a more complex biological function such as neurite outgrowth.

The importance of the isoleucine residue of the IKVAV sequence for biological activity was examined using several analogs in which the isoleucine was substituted with leucine (LAM-IL), norleucine (LAM-IX), valine (LAM-IV) and alanine (LAM-IA). LAM-IX and LAM-IV were as active for cell attachment as LAM-L. However, LAM-IL had weaker cell attachment activity than that of LAM-L and LAM-IA did not show this activity (Fig. 2C). Furthermore, LAM-IX showed approximately half of the activity for neurite outgrowth as LAM-L, and LAM-IL and LAM-IV did not promote neurite outgrowth (Fig. 2D). These results indicate that a bulky amino acid and/or β-branched side chain is important in this position for biological function.

Three synthetic peptides containing the corresponding regions of IKVAV in other laminin α chains including mouse laminin α2 chain (MER-12) [25], human laminin α3 chain (KAL-12) [26] and *Drosophila* laminin a chain (DOR-12) [27] were tested for PC12 cell adhesion and neurite outgrowth. MER-12 and DOR-12 showed cell attachment activity comparable to that of LAM-L, whereas, KAL-12 was not active (Fig. 2E). MER-12 and DOR-12 promoted neurite outgrowth as well as LAM-L (Fig. 2F). MER-12 and DOR-12 contain IKVSV and IKVGV sequences, respectively. However, in KAL-12 sequence (SKVAV), the isoleucine which is a critical residue of the IKVAV sequence for biological activity, is absent. These results are in agreement with the above structure activity study using isoleucine-substituted analogs. The coating efficiency of the synthetic peptides are 1.9% (LAM-L), 3.4% (MER-12), 0.5% (KAL-12) and 1.3% (DOR-12). Cell attachment and neurite outgrowth activities of the each peptide are similar values at 50 mg/well and 500 mg/well (Fig. 2E,F). These results indicate that differences in the peptide coating efficiencies does not significantly effect the comparison of the biological activities of the peptides within these concentrations.

In this paper, we have studied the effects of structural changes on the biological activity of the IKVAV containing 12-mer peptide. The structural requirements of the IKVAV sequence for promotion of cell attachment and neurite outgrowth were found to be very specific. The isoleucine and lysine residues are critical for biological functions but some differ-

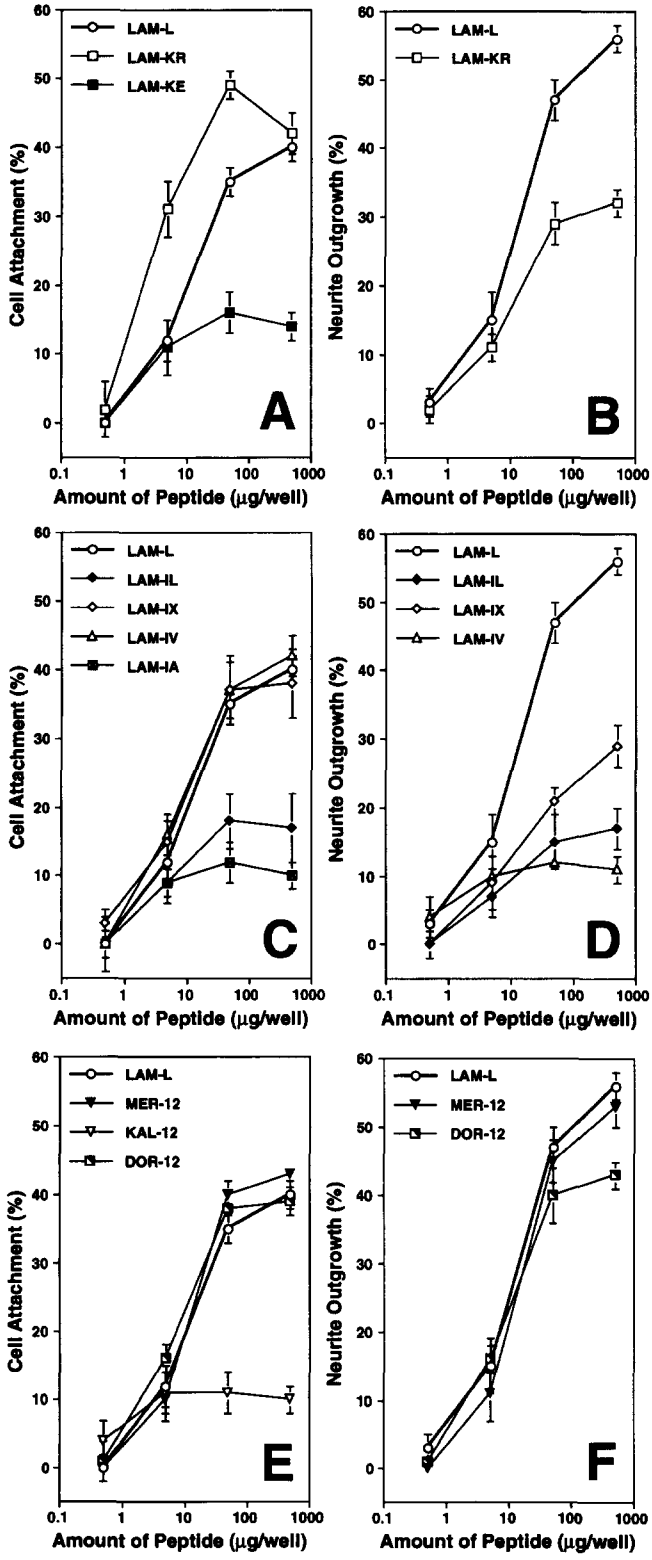

Fig. 2. Attachment (A,C,E) and neurite outgrowth promotion (B, D, F) of PC12 cells on various synthetic peptides. Each value represents the mean of three separate determinations ± S.E. Duplicate experiments gave similar results. Coating efficiencies of the synthetic peptides (500 μg/well) were 1.9% (LAM-L), 7.0% (LAM-KR), 10.4% (LAM-KE), 0.7% (LAM-IL), 2.7% (LAM-IX), 1.4% (LAM-IV), 0.4% (LAM-IA), 3.4% (MER-12), 0.5% (KAL-12) and 1.3% (DOR-12).

ences were noted in that some analogs could promote cell adhesion but had no effect on neurite outgrowth. These data suggest the possibility of more than one receptor on these cells for this domain, and that there may be different receptors depending on the cellular function. Changing the peptide structure could aid in the characterization and identification of specific receptors or binding proteins for the IKVAV sequence. In addition, identification of these structure-activity requirements is useful for designing more potent agonistic or inhibitory compounds of the IKVAV peptide for therapeutics which could be used in nerve regeneration or angiogenesis studies.

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